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## Pesticide Removal by a Mixed Culture of Bacteria and White Rot Fungi

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### Abstract

Combining activated sludge cultures with microbes harboring specific degradation pathways could constitute a relevant process for the removal of toxic and recalcitrant organic substances from wastewater. Enhanced removal of three widely used recalcitrant pesticides from their liquid mixture was demonstrated by implementing a non-acclimated mixed culture of bacteria and white rot fungus. During an incubation period of 14 days, the mixed fungus-bacteria culture achieved 47, 98, and 62% removal of aldicarb, atrazine and alachlor from the liquid phase, respectively. This compared favorably to batches containing only non-acclimated fungus or previously published removal rates with non-acclimated bacterial cultures. Biosorption along with biodegradation was responsible for the removal of the pesticides from the liquid phase. Potential application modes of the studied biodegradation process were also discussed.

*Keywords: bioaugmentation, biodegradation, biosorption, pesticide, white rot fungi*

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## 1 **1. Introduction**

2 The use of pesticides is ubiquitous in modern agriculture and is important to increase crop yield and  
3 reduce post-harvest losses. However, indiscriminate and excessive use of agricultural pesticides can  
4 lead to contamination of land and water. Emissions of pesticides come from both diffuse and point  
5 sources. The latter include mixing and loading facilities on the farm where spillages and leakages  
6 from the filling operation and spray equipment, and water from rinsing and cleaning of the  
7 equipment may contribute to pesticide contamination [1]. Wastewater generated in vegetable  
8 washing facilities and pesticide manufacturing plants are also important point sources of pollution.

9 Many pesticides are recalcitrant compounds and persist for long periods of time in the  
10 environment. Pesticides have been detected in ground- and surface waters used for potable water  
11 supply and have been linked to adverse human health effects [2]. The adverse effects of agricultural  
12 pesticide contamination extend to birds, wild animals and plants in the aquatic habitat. Because of  
13 the widespread use of pesticides in modern agricultural practice and their potential threat to the  
14 environment, the formulation of treatment methods for the removal of these pesticides from  
15 contaminated water is essential.

16 Biological treatment technologies are usually inexpensive and effective for a range of  
17 organic contaminants. However, owing to the recalcitrant structures of the pesticides, only specific  
18 bacterial and fungal species have been reported to be capable of degrading them [3]. Interestingly,  
19 in the soil environment, bacteria and fungi seem to adopt different but complementary metabolic  
20 pathways for the degradation of these recalcitrant pesticides [4]. For instance, the contributions of  
21 both bacteria and fungi to the degradation of a chloroaromatic fungicide - chlorothalonil - in soil  
22 were confirmed by a selective inhibition method [5].

23

24 To date, the number of studies investigating novel treatment techniques for the removal of  
25 pesticides from contaminated agricultural wastewater remains limited. The bacteria-dominated  
26 conventional activated sludge process has been proved to be ineffective for pesticide removal.

27 While the importance of a mixed microbial community to initiate and complete pesticide removal  
28 in the soil environment has been convincingly demonstrated by several researchers, studies  
29 concerning the removal of pesticides from water/wastewater have been predominantly focused on  
30 selected bacterial or fungal species separately [6]. For instance, white rot fungi have been  
31 demonstrated to possess excellent capacity to initiate degradation of a wide variety of pesticides by  
32 means of their nonspecific extracellular enzyme systems. A few studies have explored the  
33 bioaugmentation synergy of enriched bacterial cultures with the conventional activated sludge [7-  
34 8]. However, there appears to be no report on the combination of fungi and bacteria to remove  
35 pesticide from the aqueous phase.

36 With the aim to address the important knowledge gap illustrated above, in this study the  
37 removal of three pesticides from their mixture using a mixed culture of bacteria and white rot fungi  
38 was investigated. The removal efficiency of the dissolved parent compounds were compared to a  
39 culture with only white-rot fungi and to previously published values with bacterial cultures. The  
40 importance, limitations, and potential application modes of the studied biodegradation process were  
41 also discussed.

## 42 **2. Materials and methods**

### 43 *2.1 Selected pesticides*

44 Three pesticides, namely, aldicarb, atrazine, and alachlor were selected in view of their  
45 widespread use and reported linkage to water pollution. Aldicarb is an N-methyl carbamate; it is an  
46 insecticide/ nematocide used on a wide range of crops including cotton, potatoes, sugarcane, and is  
47 applied as granules to the soil [3]. Atrazine is a triazine and is used as a herbicide on, for example,  
48 forests, grass, corn, and sorghum. Alachlor is a chloroacetanilide and also used as a herbicide on,  
49 for example, corn and beans. Stock solutions (500 mg L<sup>-1</sup>) of each of the three pesticides were  
50 prepared in pure methanol.

### 51 *2.2 Preparation of bioaugmented culture and nutrient medium*

52 The white-rot fungus *Coriolus versicolor*, NBRC 9791 obtained from the NITE Biological  
53 Resource Center (NBRC), Japan was used for this study. Full-grown and enzyme secreting large  
54 flocs of fungi [9] were disintegrated into fine grains by sonication (Branson sonifier 450, CT, USA)  
55 for 5 minutes under sterile condition. The resulting biomass was then incubated for a week into 500  
56 mL beakers each containing 100 mL growth medium at the optimum growth temperature of 28 °C  
57 on a shaker (BR-300LF, Taitec rotary bio-shaker, Japan) at 80 rpm. At the end of the incubation  
58 period approximately 0.5 cm granules exhibiting an enzymatic activity of approximately 30  $\mu\text{M}$   
59 substrate  $\text{min}^{-1}\text{g}^{-1}$  were obtained. On the other hand, activated sludge sample collected from  
60 Shibaura wastewater treatment plant, Tokyo, Japan was utilized for obtaining the mixed culture.  
61 The sludge was centrifuged under 2150 x g and reconstructed with Milli-Q water. The  
62 bioaugmented culture was obtained by adding equal amounts (0.05 g, dry wt.) of pure fungus  
63 granules and reconstituted activated sludge. Neither the mixed bacterial culture nor the fungus was  
64 acclimatized before incubation with pesticide.

65 A nutrient-sufficient growth medium previously optimized by Kapdan *et al.* [10] for *C.*  
66 *versicolor* was modified by adding a lesser amount of glucose ( $1 \text{ g L}^{-1}$ ) and using ammonium  
67 nitrate ( $0.13 \text{ g L}^{-1}$ ) as the nitrogen source instead of urea. The initial pH of the media was 4.5.

### 68 2.3 Batch test description

69 The test solution was prepared by adding specified amounts from each of the pesticide stock  
70 solutions to autoclaved ( $121 \text{ }^\circ\text{C}$ , 0.2 MPa, 15 min) empty beakers (8.5 cm diameter, 500 mL  
71 capacity), allowing the methanol to evaporate, and then adding 100 mL of the autoclaved nutrient  
72 medium. The final concentration of each of the pesticides in the mixture was  $10 \text{ mg L}^{-1}$ . The  
73 bioaugmented culture (0.1 g (dry weight); reconstructed activated sludge: fungus = 1:1) was  
74 aseptically transferred to the test solution. The beakers were loosely covered with aluminium foil  
75 and then placed under  $28 \text{ }^\circ\text{C}$  on a shaker at 80 rpm. Beakers containing only fungus (0.1 g) and  
76 only activated sludge (0.1 g) were also incubated in order to assess the atrazine removal  
77 performance by single cultures. Same amount of autoclaved fungus and bacteria were incubated

78 separately to assess the extent of atrazine removal owing to biosorption only. Atrazine was added  
79 into the nutrient media at a concentration of  $1200 \pm 200 \mu\text{g L}^{-1}$  during the experiments with single  
80 cultures. The batch tests were carried out in duplicate and each batch test lasted 14 days.

#### 81 *2.4 Analytical methods*

82 The pesticide concentrations were measured using HPLC coupled to a diode array detector  
83 (Hewlett Packard, 1100 series). The compounds were separated on a Novapak C18, 60 Å, 4  $\mu\text{m}$ , 3.9  
84 x 150 mm column with an acetonitrile / water eluent at the wavelength of 220 nm (atrazine) and  
85 205 nm, respectively. Pesticide concentrations above  $0.05 \text{ mg L}^{-1}$  could be quantified. Total organic  
86 carbon (TOC) and total nitrogen (TN) concentrations were measured with a TOC-TN analyzer  
87 (TOC-V, Shimadzu, Japan). The activity of fungal enzyme (Laccase) was measured by monitoring  
88 the change in absorbance at 468 nm due to the oxidation of the substrate (2,6-dimethoxyphenol) by  
89 enzyme at room temperature over a 2 min period as described in a previous study [9].

### 90 **3. Results and discussion**

#### 91 *3.1 Pesticide removal*

92 Figure 1a depicts the reduction of aqueous phase concentration of each pesticide from the  
93 mixture containing the unacclimated fungus-bacteria and fungus-only cultures. Within 7 days, the  
94 concentrations of aldicarb, atrazine, and alachlor dropped by 82, 77, and 67%, respectively, in the  
95 mixed cultures, and by 79, 81, and 59% in the fungus-only cultures. In comparison, in a previous  
96 study, we reported the removal efficiencies of these pesticides by an unacclimated bacterial culture  
97 under similar experimental conditions to be only 17, ~0, and 35% for aldicarb, atrazine, and  
98 alachlor, respectively (Modin et al., 2008).

#### 99 **[Figure 1]**

100 The concentration of all three pesticides in the fungus-only batches increased after day 7 of the  
101 incubation period. Results reported in Figure 1a suggests that the pesticides adsorbed temporarily  
102 onto the fungus biomass and later got released back to the aqueous phase. It is noteworthy that the  
103 selected pesticides are moderately hydrophobic. Hydrophobic interaction along with other  
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104 mechanism such as ion exchange, surface complexation and hydrogen bonding can play a  
105 significant role in sorption of these compounds to solid substrate. Therefore, in good agreement  
106 with several previous studies [11], biosorption of the selected pesticides to the fungus biomass is  
107 not entirely unexpected. However, the observed increase in liquid phase concentration at the later  
108 stage may be attributed to desorption of the non-biodegraded portion of the initially biosorbed  
109 pesticides.

110 A different trend was observed in the fungus-bacteria mixtures. The aldicarb concentration  
111 increased slightly suggesting release by the biomass after day 7. Atrazine, on the other hand, was  
112 almost completely removed from the aqueous phase. The alachlor concentration remained steady  
113 between day 7 and day 14. The high removal of atrazine by the fungus-bacteria mixture is  
114 especially interesting since among the three tested pesticides, atrazine is generally considered the  
115 most persistent with a half-life typically over 100 days [12]. In contrast, in this study the removal of  
116 atrazine by the bioaugmented culture reached over 98% in two weeks. In additional experiments,  
117 where the extent of biosorption and biodegradation was assessed separately, it was confirmed that  
118 biodegradation of atrazine did not occur in fungus-only and activated sludge-only cultures (Figure  
119 1b).

120 The success of the fungus-bacterial mixture for removal of atrazine and alachlor can be  
121 explained in analogy to a relevant observation made in the soil environment by Levanon [13]. It  
122 was reported that the mineralization of alkyl-side chains of alachlor and alkyl-amino-side chains of  
123 atrazine was mainly due to fungal activity. However, neither heterocyclic ring-labelled atrazine nor  
124 aromatic ring-labelled alachlor were degraded when fungi or bacteria were separately inhibited.  
125 The importance of combining fungi and bacteria can be further recognized by comparing the  
126 removal rates observed in this study with that by individual bacterial and fungal species or enriched  
127 mixed bacterial cultures reported in the literature. For instance, the fungus *C. versicolor* was  
128 reported to take 42 days to reach the maximum 86% degradation of atrazine in liquid culture [14].  
129 Knapp et al. [15] reported a half life ranging 16-122 days of alachlor in surface water environment.



130 While the culture conditions can be significantly different in individual studies, thereby restricting  
131 direct comparison of removal efficiencies, it can generally be stated that the mixed fungus-bacteria  
132 culture implemented in this study exhibited high pesticide removal capacity, despite not being  
133 acclimated to the pesticides. In practice, the possibility to use non-acclimated microbial cultures for  
134 pesticide degradation may be beneficial since the use of pesticides, and thus the pesticide load in  
135 wastewater, vary significantly during a year.

### 136 *3.2 Fungi morphology and extent of biosorption*

137 For the fungus-bacteria mixture, the initial distinct fungus granules appeared disintegrated  
138 at the end of the incubation period. Disintegration, but to a lesser extent, was also observed when  
139 the fungus was incubated alone (Figure 2). These results show that the presence of bacteria had a  
140 clear effect on the morphology of the fungus.

#### 141 **[Figure 2]**

142 The mechanisms of pesticide removal from the liquid phase in this study are not entirely  
143 clear. Physical adsorption or uptake by the biomass without biochemical degradation of the  
144 pesticides appears to be a major removal mechanism by the fungus-only cultures. The pesticides  
145 were initially taken up by the fungus but later released, perhaps as a result of the change in  
146 morphology (Figure 2) and also the depletion of nutrients in the growth medium as demonstrated  
147 by the TOC and TN concentration over the incubation period (Figure 3).

#### 148 **[Figure 3]**

149 For the fungus-bacteria mixed cultures, biochemical degradation and physical  
150 adsorption/uptake both appears to have played roles for removing pesticides from the liquid phase.  
151 In the case of aldicarb, there is a slight increase in concentration between day 7 and day 12.  
152 However, for atrazine there is a clear decrease to nearly non-detectable levels, and for alachlor the  
153 concentration remains stable between day 7 and day 14 (Figure 1a). This suggests that it was not  
154 biosorption alone that was responsible for the pesticide removal, but biochemical degradation also  
155 played a role, at least for atrazine. No biodegradation or biosorption of atrazine was noticed in

156 bacteria-only culture. On the other hand, temporary adsorption of atrazine onto fungus was  
157 observed, but again no biodegradation was detected in fungus-only culture (Figure 1b). Therefore  
158 synergistic biodegradation of atrazine by the fungus-bacteria mixed culture following its initial  
159 biosorption appears to be very likely. Thus despite comparatively more pronounced disintegration  
160 of the biomass as well as the reduction of residual nutrient levels in test solution (Figures 2 and 3)  
161 the bioaugmented culture demonstrated better removal.

162 As described in section 3.1, the pesticide removal rate by the fungi-bacteria culture was  
163 high compared to the previously published values by our group [12] for bacterial cultures under  
164 similar conditions. The results from the current study suggest that the presence of bacteria has a  
165 clear effect on the fungus morphology (Figure 2) and nutrient depletion from the test solution  
166 (Figure 3). Further research is necessary to establish how bacteria affect fungus morphology and  
167 fungal activity (enzyme secretion). For further insight into the reported results, the kinetics of  
168 pesticide removal and its toxic effect on bioaugmented culture need to be studied under a range of  
169 pesticide dosage and varying ratios of fungus and activated sludge masses. Nevertheless, the results  
170 presented in the current study do suggest that combining fungus and bacteria for removing  
171 recalcitrant compounds such as pesticides from wastewater is beneficial.

#### 172 **4. Conclusions**

173 This study showed that mixed cultures of bacteria and fungus can remove the pesticides  
174 aldicarb, atrazine, and alachlor from their mixture. Particularly atrazine was very effectively  
175 removed. The reported data demonstrated the complementary effect of combining bacteria and a  
176 fungal culture for improving the removal rates of the three selected recalcitrant pesticides. The  
177 results have important implications towards the development of a scaled-up treatment system for  
178 pesticide contaminated water.

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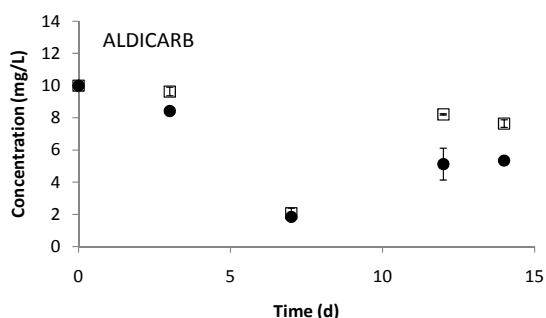
## FIGURES

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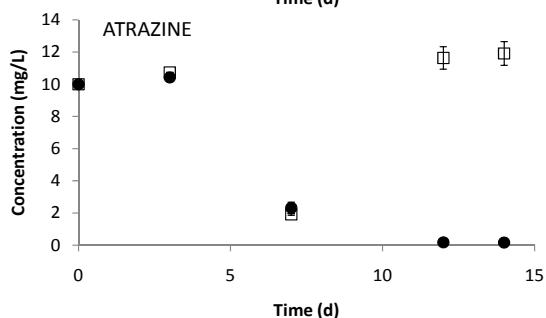
(a)

Fungus □ Fungus-bacteria ●

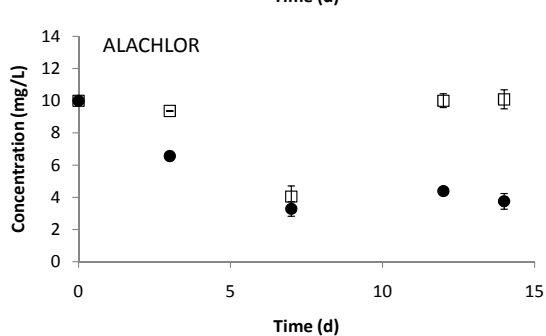
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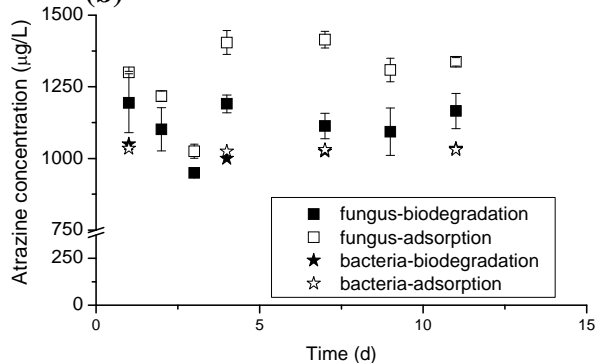


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(b)



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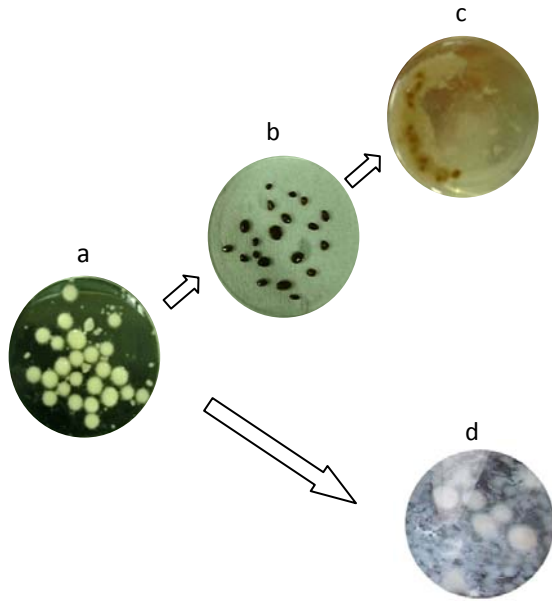
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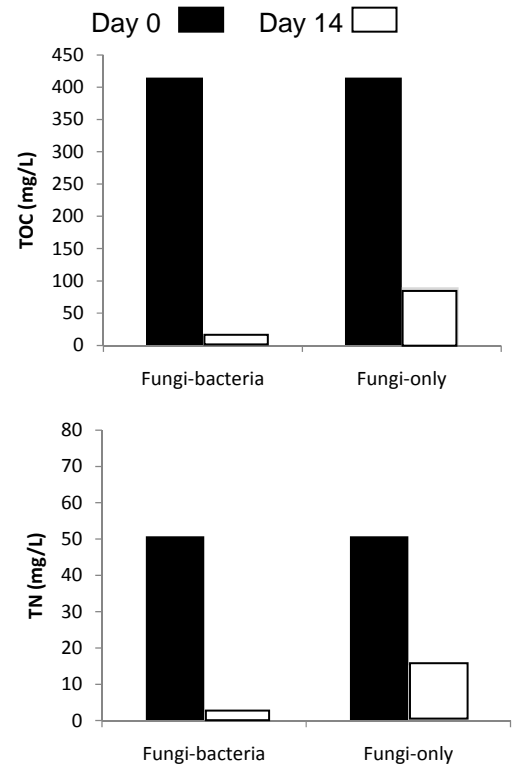
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**Figure 1.** Change in liquid phase concentration of the pesticide(s) over the incubation period. Error bars indicate the deviations between two replicates. (a) The batch test solution comprised of three pesticides (each at  $10 \text{ mg L}^{-1}$ ) and a nutrient medium. (b) The test solution comprised of atrazine ( $1200 \pm 200 \text{ } \mu\text{g L}^{-1}$ ) and a nutrient medium. Controls inoculated with heat-killed (autoclaved) biomass were added to assess biosorption separately.

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**Figure 2.** Difference between change of morphology of the fungus-only and the fungi-bacteria culture over the incubation period. (a) Firm pure fungus granules at the start of the experiment (b) Fungus granules appearing black due to being covered with activated sludge (day 3 of incubation of mixed culture batches). (c) Disintegrated fungus granules (smaller in size and deformed) still covered with activated sludge at the end of the mixed culture incubation. Presence of significant amount of fibrous structures along with granules noticeable. (d) Fungus granules (relatively more intact as compared to 'c') along with some fungus in fibrous morphology at the end of the fungus-only incubation.



**Figure 3.** Concentration of TOC and TN in the batch solutions at the start and the end of the experiment showing the effect of presence of bacteria on consumption of TOC and TN. The batch test solution comprised of three pesticides and a nutrient medium.